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If Orchid Mycorrhizal Fungi Are So Specific, How Do Natural Hybrids Cope?

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Abstract. The genus *Caladenia* (spider orchids) is one of the most diverse in southwest Western Australia, and *Caladenia* species have among the most specific mycorrhizal relationships known in the orchid family. *Caladenia* species, however, also hybridize frequently and prolifically in nature. This study considered five natural hybrids within *Caladenia* and its closest relatives to elucidate the issue of mycorrhizal specificity in the hybrids and their parental species. Symbiotic cross-germination studies of parental and hybrid seed on fungi from the species and the naturally occurring hybrids were compared with data from genetic fingerprinting (amplified fragment length polymorphism, AFLP) studies of the fungi. The germination study found that, while hybrid seeds can utilize the fungi from either parental species under laboratory conditions, it is likely that the natural hybrids in-situ share the fungus of one parent only. In contrast, the genetic analysis indicated that while the parental species always possessed genetically distinct fungal strains, the hybrids may share the mycorrhizal fungus of one parental species or possess a genetically distinct fungal strain, which is more closely related to the fungus of one parental species than the other. These findings confirm the specificity of mycorrhizal relationships in *Caladenia* and suggest the potential of hybridization and the utilization of novel fungi as a possible pathway to speciation.

Key words: terrestrial orchid, hybrid, mycorrhizal fungi, specificity, symbiotic germination, amplified fragment length polymorphism (AFLP)

Introduction

Orchidaceae is the most species-rich flowering plant family in the world, with estimates of up to 35,000 species, including both terrestrial and epiphytic life forms (Batty et al. 2002). Orchids form mycorrhizal associations with specialized soil fungi, and terrestrial orchids in particular may be heavily dependant on their fungal associates for mineral nutrition and as an energy source (Rasmussen 1995, 2002; Batty et al. 2002). Orchid seeds are extremely small and contain very little food reserves for germination (Rasmussen 1995, Batty et al. 2002). Symbiotic germination of terrestrial orchid seeds is much more rapid and effective than asymbiotic germination, and in some cases, germination can only be achieved by symbiotic means (Rasmussen 1995, Batty et al. 2002). Specificity of orchid mycorrhizal associations varies but tends to be high in the terrestrial orchids of Australia (Warcup 1981, Ramsay et al. 1986, Batty et al. 2002). Gaining an understanding of orchid mycorrhizal relationships and specificity is essential for conservation efforts to be fully effective (Batty et al. 2002, Ramsay & Dixon 2003).

This research focused on *Caladenia* hybrids. The genus *Caladenia* (spider orchids) is one of the most diverse in southwest Western Australia, with more than 110 species occurring in this region of Mediterranean-type climate (Hoffman &

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TABLE 1. *Caladenia* hybrids studied, their parental species, hybrid names, and relevant subgenera.

Caladenia hybrid parental species	*Caladenia* hybrid name	*Caladenia* subgenus
C. falcata (Nicholls) M.A. Clem. & Hopper × *longicauda* Lindl.	*C.* × *cala* Hopper & A.P. Br.	*Calonema* (Lindl.) Hopper & A.P. Br.
C. flava R. Br. × *latifolia* R. Br.	*C.* × *spectabilis* Hopper & A.P. Br.	*Elevatae* Hopper & A.P. Br.
C. flava R. Br. × *longicauda* Lindl.	*C.* × *triangularis* R. Rogers	*Elevatae* Hopper & A.P. Br. × *Calonema* (Lindl.) Hopper & A.P. Br.
C. chapmanii Hopper & A.P. Br. × *longicauda* Lindl.	*C.* × *eludens* Hopper & A.P. Br.	*Phlebochilus* (Benth.) Hopper & A.P. Br. × *Calonema* (Lindl.) Hopper & A.P. Br.
Elythranthera brunonis (Endl.) A.S. George × *Cyanicula sericea* (Lindl.) Hopper & A.P. Br.	× *Cyanthera glossodioides*	Intergeneric (*Caladenia* allies that were once included in *Caladenia*)

Source: Hoffman and Brown (1998), Hopper and Brown (2001).

Brown 1998, Hopper & Brown 2001). *Caladenia* species have among the most specific mycorrhizal relationships known in the orchid family (Warcup 1981, Ramsay et al. 1986). *Caladenia* species, however, also hybridize frequently, depending on the closeness of the relationship between the parental species, and self-perpetuating hybrid lineages may develop (Hoffman & Brown 1998, Hopper & Brown 2001). All but one of the more than 110 species of *Caladenia* found in southwest Western Australia have been observed to form natural hybrids (R. Heberle pers. comm.), many of which are fertile and some of which are quite common (Hopper & Brown 2001). If *Caladenia* species have such specific mycorrhizal relationships, how do the natural hybrids survive? Do the hybrids use one parental fungus, both parental fungi, or a completely different fungus?

Caladenia is divided into five subgenera (Hopper & Brown 2001): *Caladenia* (one species only in southwest Western Australia), *Calonema* (55 endemic species in the southwest), *Drakonorchis* (four endemic species), *Elevatae* (five species in the southwest, four of which are endemic), and *Phlebochilus* (45 species in the southwest, all but three endemic). The most common hybrids occur within a subgenus, and hybrids between different subgenera are less common (Hopper & Brown 2001).

Of the five hybrids studied here to elucidate mycorrhizal specificity (TABLE 1, FIGURE 1), two combinations were relatively common and occurred within a subgenus, two were rare and occurred between different subgenera, and one was an extremely rare intergeneric hybrid, occurring between two genera that once were included in *Caladenia* (Hopper & Brown 2001).

MATERIALS AND METHODS

Seed and Inoculum Sources

The locations of the sites from which the hybrids and their parental species were collected are shown in FIGURE 2, and a summary of seed and inoculum sources in TABLE 2.

Kings Park and Botanic Garden (KP)

The site for *Caladenia flava* × *latifolia* was Kings Park and Botanic Garden, which is located in the center of Perth and consists of substantially modified *Eucalyptus-Banksia-Allocasuarina* woodland. In September 2001, two hybrid plants of *C. flava* × *latifolia* were discovered flowering in the park. These two plants, and two individuals from each parental species from the same area, were collected and their fungi isolated.

Brookton Highway (B)

Caladenia falcata × *longicauda* was found at the Brookton Highway site, a weedy road reserve in *Eucalyptus loxophleba-Acacia acuminata* woodland ca. 100 km east of Perth. In September 2000, three individuals of the hybrid *C. falcata* × *longicauda* and three individuals of each parental species were collected and their fungi isolated. Hand pollinations were carried out to produce capsules of parental seed and of hybrid seed of both maternal types.

Medina (M)

Caladenia flava × *latifolia* was found at Medina, which is ca. 30 km south of Perth and consists of relatively pristine *Eucalyptus-Banksia-Allocasuarina* woodland. In October 2000, the hybrid *C. flava* × *latifolia* was reported to have been found at the site. Subsequent searches

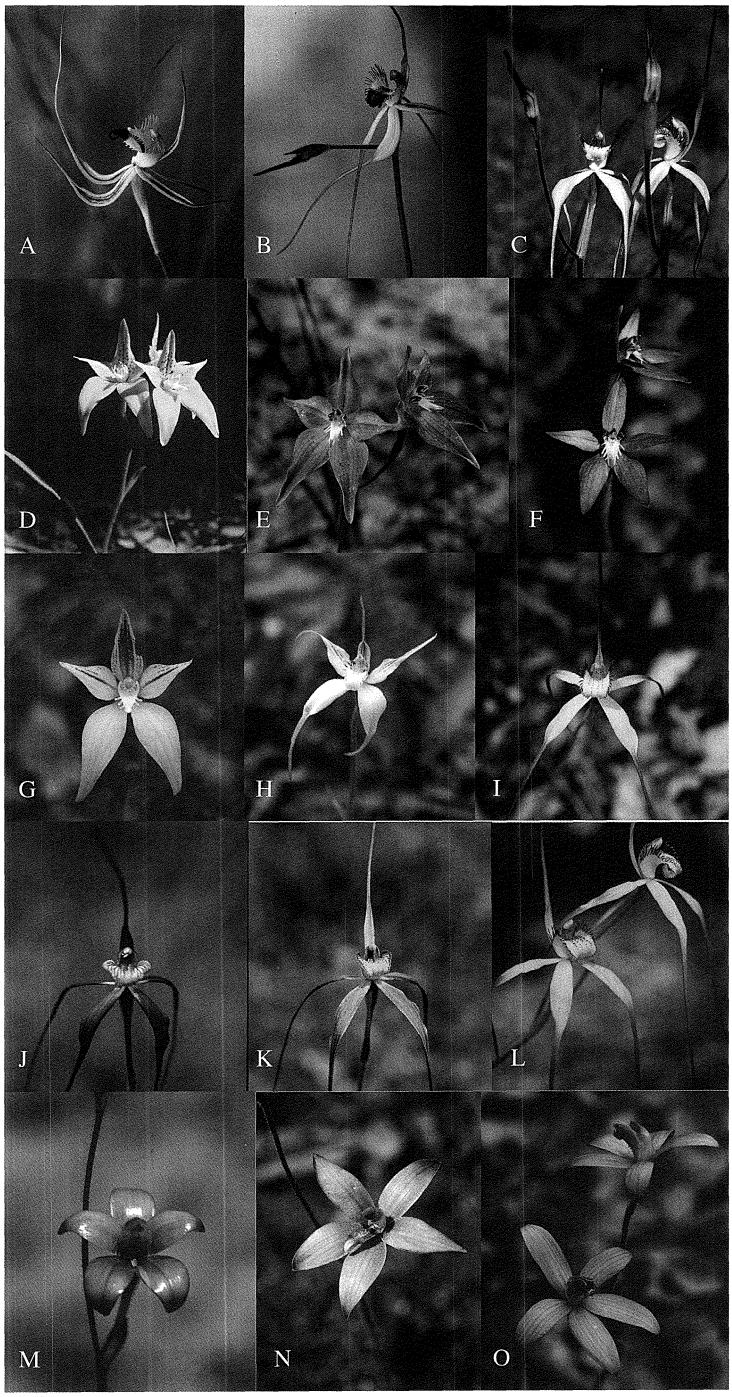

FIGURE 1. *Caladenia* hybrids and their parental species. **A.** *Caladenia falcata.* **B.** *Caladenia falcata* × *longicauda* (*C.* × *cala*). **C.** *Caladenia longicauda.* **D.** *Caladenia flava.* **E.** *Caladenia flava* × *latifolia* (*C.* × *spectabilis*). **F.** *Caladenia latifolia.* **G.** *Caladenia flava.* **H.** *Caladenia flava* × *longicauda* (*C.* × *triangularis*). **I.** *Caladenia longicauda.* **J.** *Caladenia chapmanii.* **K.** *Caladenia chapmanii* × *longicauda* (*C.* × *eludens*). **L.** *Caladenia longicauda.* **M.** *Elythranthera brunonis.* **N.** *Elythranthera brunonis* × *Cyanicula sericea* (× *Cyanthera glossodioides*). **O.** *Cyanicula sericea.* Photo credits: A–C, Gary Brockman; D–F and L–O, Andrew Brown; and G–K Nicolas Alleonard.

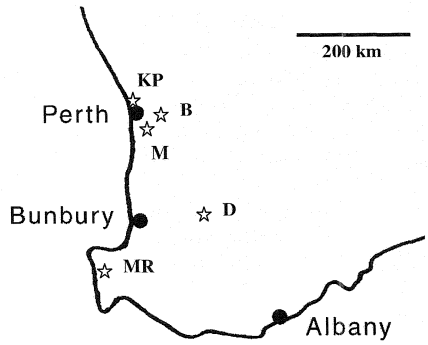

FIGURE 2. Collection locations for *Caladenia* hybrids: KP = Kings Park and Botanic Garden (*C. flava* × *latifolia*), B = Brookton Highway (*C. falcata* × *longicauda*), M = Medina (*C. flava* × *latifolia*), D = Darkan (*C. flava* × *longicauda, C. chapmanii* × *longicauda*), and MR = Margaret River (*C. flava* × *latifolia, Elythranthera brunonis* × *Cyanicula sericea*).

failed to locate the hybrids, but three individuals of each parental species were collected from an area where both were abundant, and their fungi were isolated. Hand pollinations were carried out to produce capsules of parental seed and of hybrid seed of both maternal types.

Darkan (D)

Caladenia flava × *longicauda* and *Caladenia chapmanii* × *longicauda* were found at the Darkan site, which is ca. 200 km southeast of Perth and consists of relatively unmodified *Eucalyptus wandoo* woodland. Two *Caladenia* hybrids were found there in September 2000. Two individuals of *C. flava* × *longicauda,* three of *C. chapmanii* × *longicauda,* and three individuals of each parental species (five of *C. longicauda*) were collected from the site, and their fungi were isolated. Hand pollinations were carried out to produce capsules of parental seed and of hybrid seed of all four maternal types.

Margaret River (MR)

The site for *Caladenia flava* × *latifolia* and *Elythranthera brunonis* × *Cyanicula sericea* was Margaret River ca. 300 km south of Perth. The two hybrids from this area were collected from separate sites, both of which consisted of substantially modified *Eucalyptus diversicolor* forest. The first site is ca. 50 km southeast of Margaret River, on private land used for cattle grazing, and has a large hybrid lineage of *Caladenia flava* × *latifolia.* In October 2000, three individuals of the hybrid and three individuals of each of the parental species were collected from the site, and their fungi were isolated. The second site is ca. 5 km west of Margaret River, on a road reserve. The extremely uncommon *Elythranthera brunonis* × *Cyanicula sericea* was found there in October 2000. The single hybrid plant and three individuals of each parental species were collected, and their fungi were isolated.

Symbiotic Germination

The mycorrhizal fungi were isolated by the single peloton isolation method described in Ramsay and Dixon (2003), involving the rinsing and plating out of single pelotons isolated from shredded mycorrhizal tissue by glass capillary tube collection. Symbiotic seed germination was carried out according to the methods described by Ramsay and Dixon (2003), with seeds encased in filter paper packets, sterilized for 30 min. in 1% calcium hypochlorite, and placed on oatmeal agar (2.5 g l^{-1} oatmeal, 8 g l^{-1} agar, pH 5.5) previously inoculated with the appropriate fungus.

For each hybrid, each of the available seed stocks (hybrid and parental species) was tested against each of the fungal isolates from the hybrid and the parental species in a symbiotic germination matrix. Where available, seed stocks

TABLE 2. Seed and inoculum sources for the studied *Caladenia* hybrids.

Caladenia hybrid	Source	
	Seed	Inoculum
C. falcate × *longicauda*	Brookton Highway (B)	Brookton Highway (B)
C. flava × *latifolia*	Medina (M)	Medina (M) (parental species only)
	Kings Park and Botanic Garden (*C. flava* only)	Margaret River (MR) Kings Park and Botanic Garden (KP)
C. flava × *longicauda*	Darkan (D)	Darkan (D)
	Kings Park and Botanic Garden (*C. flava* only)	
C. chapmanii × *longicauda*	Darkan (D)	Darkan (D)
Elythranthera brunonis × *Cyanicula sericea*	Roleystone (*E. brunonis* only)	Margaret River (MR)

from the same location as the fungal isolates were used. When more than one seed type was tested against a fungal isolate, they were tested in the same petri dish, on separate pieces of filter paper. Germination was scored, at 12 weeks after sowing, as the number of seedlings that had reached or passed the stage of trichome development, as a percentage of viable seed (i.e., non-imbibed seeds were not included). The percentage of nonviable seed differed between but not within each seed type. An asymbiotic control was used for each experiment. Fungi that did not support germination to the stage of trichome development of any of the seed types tested were considered non-mycorrhizal and were not included in the amplified fragment length polymorphism (AFLP) analysis.

DNA Extraction

Fungal isolates for DNA extraction were grown in vegetable juice (Campbell's V8 vegetable juice diluted to $\frac{1}{5}$ and autoclaved, see Pope & Carter 2001), with sufficient biomass for DNA extraction produced after 3–4 weeks. The fungal mycelium was frozen with liquid nitrogen without grinding, followed by use of the Qiagen DNeasy® Plant Mini Kit according to the manufacturer's instructions.

Amplified Fragment Length Polymorphism

DNA fingerprints were produced using Amplified Fragment Length Polymorphism (AFLP), adapted from the four-step process described in Krauss and Hopper (2001).

(1) Restriction Digest of the DNA. Approximately 200–250 ng of DNA was digested with 1 μL *EcoRI/MseI* restriction enzyme in a reaction volume of 12.5 μl per sample, incubated at 37°C for 2 hours, then at 70°C for 15 min. before cooling on ice.

(2) Ligation of Adapters. 0.5 μl of T4 DNA ligase and 12 μl of adapter/ligation solution were added to each digested sample, which were then incubated at 20°C overnight.

(3) Pre-Selective Amplification by PCR. 1.25 μl of the ligation mix was added to 10 μl of pre-amplification primer mix II, 1.25 μl of 10× PCR buffer for AFLP, 0.1 μl of *Taq* DNA polymerase, 0.375 μl of $MgCl_2$, and 0.375 μl of water in a PCR plate. PCR was performed for 20 cycles of 94°C for 30 sec., 56°C for 2 min., and 72°C for 2 min. The pre-amplification product was diluted 1:30 in 0.1 M TE buffer.

(4) Selective Amplification by PCR. A total of 1.25 μl of each diluted pre-selective DNA sample was added to 1 μl of 10× PCR buffer, 0.05 μ of *Taq* DNA polymerase, 0.3 μl of $MgCl_2$, 3.4 μl of water, 0.2 mM of each of four dNTPs, and primers (the fluorescently labeled 3 base extension *EcoRI* primers e-act, e-agg, and e-acc; the 2 base extension *MseI* primer m-cc). PCR was performed for 3 cycles of 94°C for 10 min., 70°C for 2 min., 72°C for 2 min., then 8 touchdown cycles of 94°C for 30 sec., 69°C for 2 min., 72°C for 2 min., then 23 cycles of 94°C for 30 sec., 61°C for 2 min., 72°C for 2 min., followed by a 60°C hold for 30 min.

All AFLP reagents were purchased in kit form from Invitrogen (Carlsbad, CA, USA). PCR was performed on an Applied Biosystem GeneAmp® 9700 Thermocycler. The fluorescently labeled amplified fragments were analyzed by gel electrophoresis (6% polyacrylamide gels) by an ABI Prism 377 Automated DNA Sequencer. Fragments were sized through the inclusion of GeneScan Rox 500 internal size standard (Applied Biosystems, Warrington, UK). Multi-locus profiles were visualized by ABI GeneScan software (Applied Biosystems, Foster City, USA). AFLP profiles were scored for the presence/absence of fragments 50–500 base pairs in size.

Statistics

Seed germination data were statistically analyzed by ANOVA. Fisher's PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

A principal coordinates analysis (PCA) based on genetic distance (Euclidean distance) was performed on each set of AFLP fingerprints to illustrate the pattern of genetic variation within and between fungal source species, using the genetic analysis program GenAlEx (Peakall & Smouse 2001).

Results

Caladenia falcata × *longicauda* (*C.* × *cala*)

Symbiotic* Germination

The *Caladenia longicauda* and hybrid seeds had very similar patterns of germination across all fungal isolates (Table 3). Both seed types germinated well on all fungal isolates except isolate *C. falcata* 3b, which was nonmycorrhizal. The *C. falcata* seed only germinated on isolates *C. falcata* 1b and 2c (Table 3). All three

* No seeds of any parental species or hybrids germinated under asymbiotic conditions (data not shown).

TABLE 3. Mean percentage germination to or beyond the stage of trichome development (standard error) of *Caladenia falcata* × *longicauda* and seeds of parental species on fungal isolates from the hybrid and both parental species.

Fungal source species	Plant no.	Isolate no.[1]	Seed type[2]		
			Caladenia longicauda[C]	Hybrid[B]	*Caladenia falcata*[A]
C. longicauda	**1**	**a**^{de}	25.2 (9.39)*	11.8 (4.53)*	0 (0)
	2	a^{ab}	4.9 (4.94)	1.4 (1.42)	0 (0)
	3	b^{bcd}	11.2 (4.41)*	5.4 (2.97)*	0 (0)
Hybrid	1	a^{bc}	13.7 (3.11)*	1.1 (1.08)	0 (0)
	2	b^{cde}	18.5 (6.98)*	9.6 (2.41)*	0 (0)
	3	a^{e}	30.1 (10.32)*	8.7 (2.54)*	0 (0)
C. falcata	1	b^{f}	36.4 (8.21)*	61.0 (8.90)*	53.7 (9.80)*
	2	**c**^{g}	42.5 (3.17)*	71.1 (2.11)*	61.4 (7.52)*
	3	**b**^{a}	0 (0)	0 (0)	0 (0)

* Germination statistically significantly higher than zero ($P < 0.05$).

[1] Fungal isolates in bold were not included in the AFLP analyses. Isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types ($P < 0.05$).

[2] Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates ($P < 0.05$).

seed types produced their highest levels of germination on these two fungal isolates (*C. falcata* 1b and 2c, TABLE 3). The levels of germination were all statistically significantly higher than zero, except for those of *C. longicauda* and the hybrid on isolate *C. longicauda* 2a, and of the hybrid on isolate hybrid 1a (TABLE 3).

All fungal isolates except *Caladenia longicauda* 2a and *C. falcata* 3b supported levels of germination that were statistically significantly higher than zero when data from all seed types were bulked (TABLE 3). Isolate hybrid 3a supported a statistically significantly higher level of germination than the other *C. longicauda* and hybrid isolates across all seed types, and isolates *C. falcata* 1b and 2c supported statistically significantly higher levels of germination than any other isolates. Isolate *C. falcata* 2c had the highest level of germination across all seed types. Across all fungal isolates, the *C. longicauda* seed displayed the highest germination, and *C. falcata* the lowest (TABLE 3).

Amplified Fragment Length Polymorphism

The Principal Coordinates Analysis (PCA) of the fungal AFLP fingerprints grouped the isolates according to source species, with the isolates from the hybrid lying between those from the two parental species (FIGURE 3). None of the groups of isolates overlapped. The two isolates from *Caladenia longicauda* were closely grouped together, as were the three isolates from the hybrid. Only one isolate from *C. falcata* was included (FIGURE 3).

Caladenia flava × *latifolia* (*C.* × *spectabilis*)

Symbiotic Germination

The *Caladenia flava* × *latifolia* seed germinated very well (statistically significantly higher than zero) on most fungal isolates, the exceptions being isolates *C. latifolia* MR3b and *C. flava* KP3a and KP3b, with no germination, and isolates hybrid MR1a, KP1a, and KP2b, and *C. latifolia* KP2a with very low germination levels (statistically indistinguishable from zero) (TABLE 4). It is noteworthy that of the seven exceptions listed above, three produced no significant germination of any seed type, while the remaining four, which only germinated *C. flava* seed, were all from the KP location. The hybrid seed only germinated well on one (of six) KP fungal isolates, and that one was from *C. latifolia* (TABLE 4).

The *Caladenia flava* seed germination indicated a preference for its own or the hybrid's

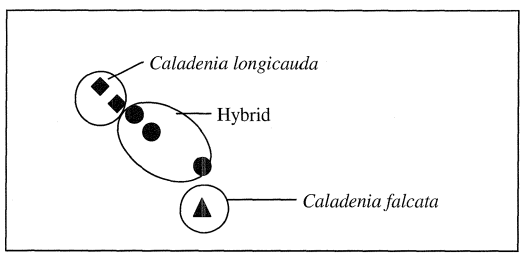

FIGURE 3. Principal Coordinates Analysis of amplified fragment length polymorphism (AFLP) fingerprints of fungal isolates from *Caladenia falcata* × *longicauda* and both parental species.

TABLE 4. Mean percentage germination to or beyond the stage of trichome development (standard error) of *Caladenia flava* × *latifolia* and seeds of parental species on fungal isolates from the hybrid and both parental species from three locations (see FIGURE 2).

Fungal source species	Loc.	Plant no.	Isolate no.[1]	Seed type[2]		
				Caladenia flava[A]	Hybrid[B]	*Caladenia latifolia*[A]
C. flava	M	1	a^{d}	30.2 (4.37)*	21.8 (8.15)*	0 (0)
		2	c^{cd}	1.1 (1.12)	38.2 (5.46)*	2.3 (2.30)
		3	$\mathbf{b}^{bc}$	5.0 (1.72)	22.4 (21.50)*	0 (0)
C. latifolia	M	**1**	$\mathbf{a}^{d}$	0 (0)	17.4 (3.40)*	33.3 (2.55)*
		2	$\mathbf{a}^{d}$	0 (0)	30.8 (17.92)*	33.7 (12.68)*
		3	a^{de}	0 (0)	15.0 (3.32)*	50.4 (1.88)*
C. flava	MR	1	b^{f}	19.9 (5.04)*	62.9 (6.96)*	17.5 (4.25)*
		2	a^{de}	19.0 (15.61)*	52.5 (3.85)*	1.1 (0.57)
		3	b^{de}	8.9 (4.49)	52.0 (5.67)*	1.0 (0.98)
Hybrid	MR	1	a^{ab}	0 (0)	6.8 (5.33)	0 (0)
			b^{d}	20.3 (5.19)*	39.5 (4.37)*	0 (0)
		3	a^{d}	16.1 (10.46)*	53.8 (8.59)*	0 (0)
C. latifolia	MR	1	a^{ef}	10.0 (3.55)*	71.5 (12.02)*	8.3 (2.12)*
		2	a^{d}	12.1 (6.69)*	48.8 (13.73)*	0 (0)
		3	$\mathbf{b}^{a}$	0 (0)	0 (0)	0 (0)
C. flava	KP	3	a^{ab}	9.0 (8.01)*	0 (0)	0 (0)
			b^{ab}	10.9 (3.37)*	0 (0)	0 (0)
Hybrid	KP	2	b^{bc}	18.3 (5.40)*	1.8 (1.75)	0.4 (0.42)
		1	a^{bc}	17.4 (2.20)*	4.3 (4.35)	0 (0)
C. latifolia	KP	2	a^{ab}	1.7 (1.67)	1.0 (0.98)	0 (0)
		1	a^{f}	22.9 (3.85)*	39.9 (1.57)*	54.4 (4.54)*

* Germination statistically significantly higher than zero ($P < 0.05$).

[1] Fungal isolates in bold were not included in the AFLP analyses. Isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types ($P < 0.05$). Fungi from KP analyzed separately.

[2] Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates ($P < 0.05$).

fungi, germinating well on five (of eight) *C. flava* isolates, four (of five) hybrid isolates, and three (of eight) *C. latifolia* isolates, and poorly on the remaining three *C. flava* isolates (M2c, M3b, and MR3b) and one *C. latifolia* isolate (KP2a) (TABLE 4). Greater specificity was evident with isolates from the M location than those from MR or KP (TABLE 4).

In comparison, the *Caladenia latifolia* seed displayed a much narrower specificity, germinating well on five (of eight) of its own fungal isolates and one (of eight) *C. flava* isolates, and poorly on three (of eight) *C. flava* isolates and one (of five) hybrid isolate (TABLE 4). Again, specificity was greatest at the M location, with the *C. latifolia* seed germinating well on all three of its own fungal isolates and poorly or not at all on the *C. flava* isolates (TABLE 4). The *C. latifolia* seed did not germinate on any of the hybrid isolates from the MR location, and poorly or not at all on five (of six) isolates from the KP location (TABLE 4).

The hybrid seed displayed statistically significantly higher germination than either parental species across all fungal isolates (TABLE 4).

It is noteworthy that three of the fungal isolates germinated all three types of seed well: isolates *Caladenia latifolia* KP1a and MR1a, and isolate *C. flava* MR1b (TABLE 4). In some cases, germination was less for the species from which the fungus had been isolated than for the hybrid or the other parental species (isolates *C. latifolia* KP2a and MR2a, and isolates hybrid KP1a and KP2b). The fungal isolates *C. latifolia* KP1a and MR1a and *C. flava* MR1b, which germinated all three seed types, supported statistically significantly higher germination than the other isolates across all seed types (TABLE 4).

Amplified Fragment Length Polymorphism (AFLP)

The AFLP fingerprints of the fungal isolates clustered in the PCA according to source species and collection location (FIGURE 4). The isolates from the KP location were distinct from those from the other locations. Within the KP isolates,

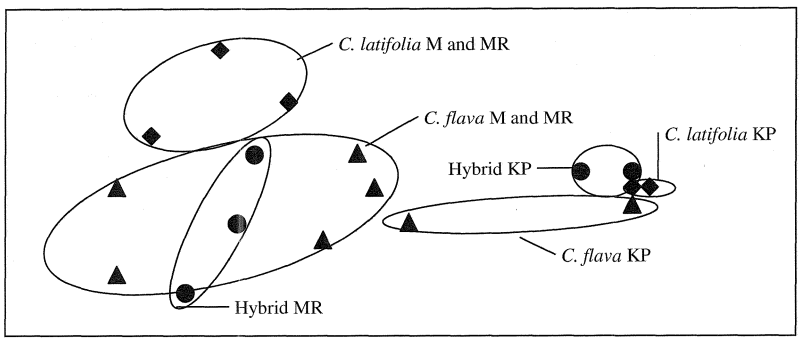

FIGURE 4. Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Caladenia flava* × *latifolia* and both parental species from three geographic regions (M = Medina, MR = Margaret River, KP = Kings Park).

grouping was according to source species, with groups of isolates very close but not overlapping. Of the two *Caladenia flava* KP isolates, one grouped closely with the other KP isolates, while the other grouped more closely to the *C. flava* isolates from the other locations. The *C. latifolia* isolates from M and MR formed distinct groups, relatively close to one another. In contrast, the *C. flava* isolates from M and MR, and the hybrid isolates from MR, all overlapped one another in a single large group (FIGURE 4).

Caladenia flava × *longicauda* (*C.* × *triangularis*)

Symbiotic Germination

The seed of the hybrid *Caladenia flava* × *longicauda* germinated well on all but one fungal isolate, *C. flava* 2a (TABLE 5). In contrast, the *C. longicauda* seed germinated on all of the hybrid and *C. longicauda* isolates, although well on only isolate hybrid 2a. The *C. flava* seed was highly specific, only germinating on its own fungi (well on isolates 1b and 1c, poorly on isolate 2a, TABLE 5).

All of the fungal isolates supported germination levels statistically significantly higher than zero across all seed types (TABLE 5). The hybrid seed displayed higher germination across all fungal isolates than did either of the parental species (TABLE 5).

Amplified Fragment Length Polymorphism (AFLP)

The isolates from *Caladenia longicauda* lay between those from the hybrid and *C. flava* in the PCA (FIGURE 5). None of the groups of isolates overlapped. The closest grouping of isolates consisted of two from *C. longicauda,* two from *C. flava,* and one from the hybrid, with the remaining isolates further from this central grouping. The two closely grouped isolates from *C. longicauda* originated from different plants, while the outlier (isolate 5c) was from the same plant as one of the other isolates. In contrast, the two closely grouped isolates from *C. flava* originated from the same plant, while the outlier (isolate 2a) was from a different plant (FIGURE 5).

Caladenia chapmanii × *longicauda* (*C.* × *eludens*)

Symbiotic Germination

The seed of the hybrid *Caladenia chapmanii* × *longicauda* only germinated well on isolate *C. longicauda* 5c, and poorly on isolate *C. longicauda* 5b (TABLE 6). In contrast, the *C. chapmanii* seed displayed specificity for its own fungi, germinating well on isolates *C. chapmanii* 2b and 3a. The *C. longicauda* seed had a wider specificity, germinating well on all of the hybrid and *C. longicauda* isolates. There was no germination of any seed type on isolate *C. chapmanii* 1a, which was non-mycorrhizal (TABLE 6).

All of the fungal isolates supported statistically significant higher germination than did isolate *Caladenia chapmanii* 1a across all seed types (TABLE 6). The *C. longicauda* seed displayed the highest germination levels across all fungal isolates, and the hybrid seeds the lowest (TABLE 6).

Amplified Fragment Length Polymorphism (AFLP)

The PCA analysis of the AFLP fingerprints grouped the fungal isolates of the parental species separately, with the isolates from the hybrid overlapping with those from *Caladenia longicauda* (FIGURE 6). The outlying isolate from *C. longicauda* (isolate 5c) was from the same plant as one of the centrally grouped isolates (FIGURE 6).

TABLE 5. Mean percentage germination to or beyond the stage of trichome development (standard error) of *Caladenia flava* × *longicauda* and seeds of parental species on fungal isolates from the hybrid and both parental species.

Fungal source species	Plant no.	Isolate no.[1]	Seed type[2]		
			Caladenia flava[A]	Hybrid[B]	*Caladenia longicauda*[A]
C. flava	1	b^{de}	16.4 (11.07)*	45.6 (16.52)*	0 (0)
		c^{cd}	7.8 (0.97)*	20.9 (4.93)*	0 (0)
	2	a^{b}	5.3 (3.22)	2.5 (2.47)	0 (0)
Hybrid	1	b^{bc}	0 (0)	11.0 (3.77)*	7.7 (6.21)
	2	a^{e}	0 (0)	50.1 (6.30)*	18.9 (6.11)*
C. longicauda	4	a^{cde}	0 (0)	43.2 (7.73)*	6.2 (3.61)
	5	b^{de}	0 (0)	59.8 (12.74)*	8.8 (5.91)
		c^{cd}	0 (0)	36.2 (11.10)*	2.8 (2.78)

* Germination statistically significantly higher than zero ($P < 0.05$).

[1] Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types ($P < 0.05$).

[2] Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates ($P < 0.05$).

Elythranthera brunonis × *Cyanicula sericea* (× *Cyanthera glossodioides*)

Symbiotic Germination

Elythranthera brunonis seed, which was the only type available for this experiment, germinated well on the hybrid isolate and *E. brunonis* 1a, and poorly on isolate *E. brunonis* 2c (TABLE 7).

Amplified Fragment Length Polymorphism (AFLP)

As only *Elythranthera brunonis* seed was available for testing, the AFLP fingerprints of nearly all of the fungi in TABLE 7 were included in the PCA. They grouped according to source species, with the isolates from the hybrid lying between those from the two parental species (FIGURE 7). Overall, the genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse 2001) between these isolates from different orchid genera was considerably greater (ca. twice as much) than between isolates from different orchid species in the same genus (*Caladenia*), results not shown.

DISCUSSION

Germination of all of the seed types used was dependent on the presence of a mycorrhizal fungus (TABLES 3–7). This confirms previous reports for a number of *Caladenia* species (Warcup 1981, Ramsay et al. 1986).

Both similarities and differences between the conclusions can be drawn from the results of the germination experiments and from the AFLP analyses. Both techniques indicated that the parental species always use different fungi from each other. The germination experiments indicated, however, that the hybrids probably share the fungi of one parental species (TABLES 3–7), while the AFLP analyses indicated that the fungi of the hybrids may be the same as those of one

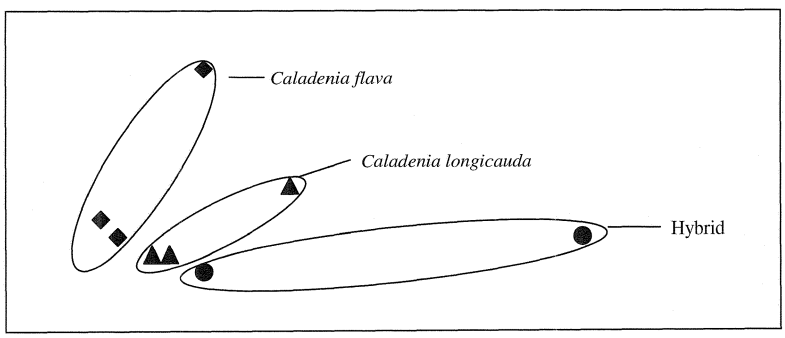

FIGURE 5. Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Caladenia flava* × *longicauda* and both parental species.

TABLE 6. Mean percentage germination to or beyond the stage of trichome development (standard error) of *Caladenia chapmanii* × *longicauda* and seeds of parental species on fungal isolates from the hybrid and both parental species.

Fungal source species	Plant no.	Isolate no.[1]	Seed type[2]		
			Caladenia chapmanii[B]	Hybrid[A]	*Caladenia longicauda*[C]
C. chapmanii	1	a[a]	0 (0)	0 (0)	0 (0)
	2	b[b]	10.0 (2.87)*	0 (0)	0 (0)
	3	a[bc]	23.4 (9.29)*	0 (0)	0 (0)
Hybrid	1	b[b]	0 (0)	—	9.6 (2.62)*
	2	c[c]	0 (0)	0 (0)	43.1 (43.08)*
	3	a[bc]	0 (0)	0 (0)	11.6 (3.92)*
C. longicauda	4	a[bc]	0 (0)	0 (0)	15.9 (5.79)*
	5	b[bc]	0 (0)	2.4 (2.38)	14.3 (10.43)*
		c[bc]	0 (0)	5.9 (3.50)*	7.5 (1.76)*

* Germination statistically significantly higher than zero ($P < 0.05$).

[1] Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types ($P < 0.05$).

[2] Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates ($P < 0.05$).

parental species or may be different from both but more closely related to one than the other (FIGURES 3–7).

It was very rare for any parental seed types to germinate on fungal isolates other than their own or those of the hybrid (TABLES 3–7), indicating that the parental species possessed different fungi from each other. This is supported by the AFLP analyses, in which each parental orchid species possessed fungal isolates that were genetically distinct from the fungal isolates of the other parental species (FIGURES 3–7).

The germination data showed that hybrid orchids might use the same mycorrhizal fungus as one parental species. *Caladenia flava* × *latifolia* probably shares the fungus of *C. flava,* as both seed types germinated on both fungal types, and this appears to be the case in all three collection locations (TABLE 4). *Caladenia flava* × *longicauda* seems to share the fungus of *C. longicauda,* as both seed types germinated on both fungal types (TABLE 5). The situation with *C. chapmanii* × *longicauda* is less clear, as the hybrid seeds only germinated on *C. longicauda* fungi (TABLE 6). The hybrid, however, would appear to share the same fungus as *C. longicauda,* because *C. longicauda* seed germinated both on its own and the hybrid's fungi. It is more difficult to reach a conclusion about *Elythranthera brunonis* × *Cyanicula sericea* because of the lack of seed of the hybrid and *C. sericea,* but it would seem probable that the hybrid shares the fungus of *E. brunonis,* as the *E. brunonis* seed germinated on the hybrid's fungus as well as its own (TABLE 7). *Caladenia falcata* × *longicauda* appears to share the fungus of *C. longicauda,* as both seed types germinated on both fungal types (TABLE 3). The issue here is complicated by the broad specificity of the mycorrhizal fungi of *C. falcata,* which germinated all three seed types.

In contrast to the germination data, the AFLP analyses showed that most of the hybrids ex-

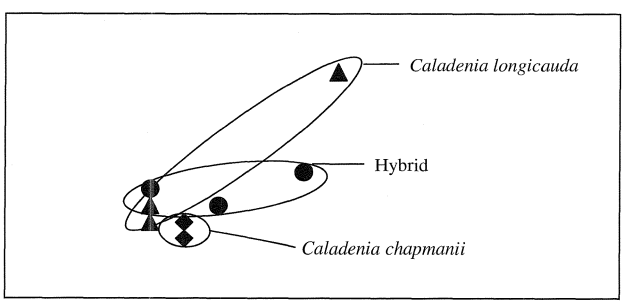

FIGURE 6. Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Caladenia chapmanii* × *longicauda* and both parental species.

TABLE 7. Mean percentage germination of *Elythranthera brunonis* seed on fungal isolates from *Elythranthera brunonis* × *Cyanicula sericea* and both parental species.

Fungal source species	Plant no.	Isolate no.[1]	To and beyond stage of trichome dev. % (SE)
Canicula sericea	1	b	0 (0)
	2	a	0 (0)
	3	**c**	0 (0)
Hybrid	1	c	4.9 (3.83)*
Elythranthera brunonis	1	a	10.8 (0.76)*
	2	c	2.3 (1.26)

[1] The fungal isolate in bold was not included in the AFLP analyses.

* Germination statistically significantly higher than zero ($P < 0.05$).

amined in this study possessed fungal isolates which were genetically distinct from those of both parental species (FIGURES 3–7). No overlap was found between the fungal isolates of the hybrids and those of either parental species for *Caladenia falcata* × *longicauda, C. flava* × *latifolia* from location KP, *C. flava* × *longicauda,* or *Elythranthera brunonis* × *Cyanicula sericea.* In two cases, however, the fungal isolates of the hybrids overlapped with those of one parental species (*Caladenia flava* × *latifolia* from location MR and *C. chapmanii* × *longicauda*), indicating that the fungal isolates of these hybrids were not genetically distinct from those of their parental species.

In all of the cases where the fungal isolates of the hybrids were genetically distinct from those of both parental species, the fungal isolates of the hybrids were more closely related to those of one parental species than the other. The fungal isolates of *Caladenia falcata* × *longicauda* were more closely related to those of *C. longicauda* than those of *C. falcata* (FIGURE 3), the fungal isolates of *C. flava* × *longicauda* were more closely related to those of *C. longicauda* than those of *C. flava* (FIGURE 5), and the fungal isolate of *Elythranthera brunonis* × *Cyanicula sericea* was more closely related to those of *E. brunonis* than those of *C. sericea* (FIGURE 7). This is supported by the Nei Genetic Distance between the hybrids and their parental species, which was always lower for the distance between the hybrid and one parental species than between the hybrid and the other parental species (data not shown).

A number of fungal isolates that could germinate both parental species, as well as the hybrid, were found; for example, *Caladenia falcata* fungi (TABLE 3), and the isolates *C. latifolia* KP1a, *C. latifolia* MR1a, and *C. flava* MR1b (TABLE 4), germinated seed of both parents and the hybrid. In other cases, a different seed type germinated much better than that of the source species, including the isolates *C. latifolia* KP2a, *C. flava* × *latifolia* KP1a, *C. flava* × *latifolia* KP2b, and *C. latifolia* MR2a (TABLE 4). These results indicate that specificity in *Caladenia* may be broader than previously thought.

The hybrid seeds appear to have a broader specificity than the seeds of their parental species. With some exceptions, the seeds of three out of the four available hybrids germinated on the fungi of both of their parental species as well as on their own (TABLES 3–5). The fourth hybrid, *Caladenia chapmanii* × *longicauda,* only germinated on *C. longicauda* fungi (TABLE 6); it is unusual for an orchid to not germinate on its own fungal isolates.

Indications are that some of the orchids studied here may use different fungi at different life stages (Rasmussen 1995, Batty et al. 2002). Some of the orchids did not germinate on their own fungi (*Caladenia latifolia* from locations MR and KP, and *C. chapmanii* × *longicauda*), suggesting that these orchids must have used a different fungus to germinate in-situ than they possessed as adult plants.

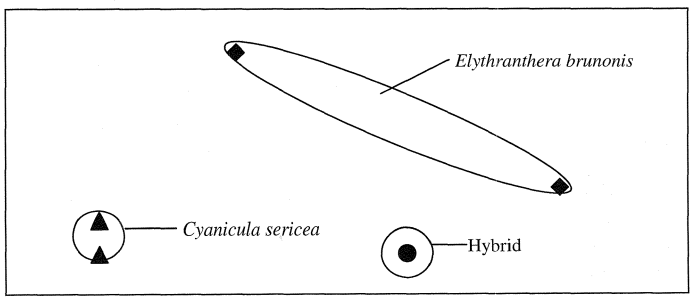

FIGURE 7. Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Elythranthera brunonis* × *Cyanicula sericea* and both parental species.

The patterns of germination specificity and of fungal genetic variation differed between locations in the one hybrid collected from more than one site (*Caladenia flava* × *latifolia*—TABLE 4, FIGURE 4). At location M, the fungal isolates were quite specific in their germination effect, while the specificity was lower at location MR, and at location KP the specificity varied between seed types. At location KP, the fungal isolates of the hybrid were genetically distinct from those of both parental species, while the fungal isolates of the hybrid from location MR overlapped with those of *C. flava* from both MR and M. These results indicate geographic as well as taxonomic influences on fungal usage and specificity. This has been suggested by Zettler et al. (2003) and clearly demonstrated in the nonphotosynthetic orchids *Corallorhiza maculata* and *C. mertensiana* by Taylor and Bruns (1999), who found that mycorrhizal fungal strain was influenced by geography and/or habitat as well as source species.

The differences between the conclusions that can be drawn from the results of the germination experiments and AFLP analyses may be related to the concepts of ecological and potential specificity (Rasmussen 2002, Fay & Krauss 2003). Ecological specificity, which is the range of fungi associated with an orchid species in-situ, is indicated by the AFLP analyses, wherein it appears that the hybrids use fungi which may or may not be genetically distinct from those of both parental species but are more closely related to the fungi of one parent than the other. Potential specificity, on the other hand, i.e., the range of fungi with which an orchid species can form viable associations in-vitro, is indicated by the germination experiments, wherein the hybrids apparently can use the fungi of both parental species as well as their own, which are likely to be the same as those of one parental species.

The availability of more fungal isolates for the AFLP analyses would have been useful. Three samples per species are minimal if firm conclusions are to be drawn from the results.

Hybridization and the utilization of fungi may be a possible path to speciation. The genus *Caladenia* is considered to be actively speciating (K. Dixon pers. comm.), and one possible path for speciation may be hybridization and the utilization of novel fungi by the hybrids. The occurrence of fertile hybrids and self-perpetuating hybrid lineages is certainly suggestive. While the avoidance of cross-pollination with either parental species would be necessary for the evolution of a hybrid into a new species, perhaps hybrids only survive (and, in particular, only form self-perpetuating hybrid lineages) where a suitable un- or under-utilized fungus occurs.

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